Instruction Manual

# diarellaMycoplasma

## real time PCR Kit

For the *in vitro* detection of the DNA of *Mycoplasma pneumoniae* in clinical specimens.

| | | |
|---|---|---|
| REF | G01089-32 | G01089-96 |
| Σ | 32 | 96 |
| - 18°C | | |

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# 1 Components

The reagents supplied are sufficient for 32 or 96 reactions respectively.

**Table 1:** Components of the diarellaMycoplasma real time PCR Kit.

| | Label | Lid Colour | Content | |
|---|---|---|---|---|
| | | | 32 | 96 |
| K1 | Reaction Mix | yellow | 1 x 515 µl | 2 x 770 µl |
| K2 | Positive Control | red | 1 x 50 µl | 1 x 100 µl |
| K3 | Negative Control | green | 1 x 50 µl | 1 x 100 µl |
| K4 | Control DNA | red | 1 x 160 µl | 2 x 240 µl |

# 2 Abbreviations

PCR Polymerase Chain Reaktion
RT Reverse Transcription

# 3 Transport and Storage

The **diarellaMycoplasma** real time PCR Kit is shipped on dry ice. All components must be stored at -18°C in the dark immediately after receipt.
Do not use reagents after the date of expiry printed on the package. After initial usage, reagents are stable for up to six months. To avoid a loss of sensitivity, the reagents should not be thawed and frozen more than two times. If necessary aliquot kit components K1, K2 and K4.

# 4 Intended Use

The **diarellaMycoplasma** real time PCR Kit is an assay for the detection of the DNA of *Mycoplasma pneumoniae* in clinical specimens (e.g. throat swabs, nasal swabs, bronchial lavage) using real time PCR microplate systems (e.g. Applied Biosystems, Stratagene, Corbett Research).

# 5 Sample Material

Starting material for the assay is DNA isolated or released from clinical specimens (e.g. throat swabs, nasal swabs, bronchial lavage).

# 6 Quality Control

In accordance with gerbion's ISO-certified Quality Management System, each lot of the **diarellaMycoplasma** real time PCR Kit is tested against predetermined specifications to ensure consistent product quality.

# 7 Product Warranty

gerbion guarantees the performance of all products when used according to the instructions given in the Instruction Manual. The purchaser must determine the suitability of the product for its particular use. Should any product fail to perform satisfactorily due to any reason other than misuse, gerbion will replace it free of charge or refund the price. We reserve the right to change, alter, or modify any product to enhance its performance and design.

# 8 Introduction

*Mycoplasma pneumoniae* is a very small bacterium which causes the disease mycoplasma pneumonia, a form of atypical bacterial pneumonia. Tracheobronchitis, laryngitis, meningitis, otitis media, and other symptoms can be caused by *Mycoplasma pneumoniae* infection. It is also associated with disorders of the haematopoietic system, the central nervous system, the liver and the pancreas, and with cardiovascular syndromes. Furthermore, a connection with asthma is suspected.

*Mycoplasma pneumoniae* does usually not occur in healthy persons, however, it is highly contagious and is transmitted by droplet infection. Children are mainly at risk.

# 9 Principle of the Test

The **diarellaMycoplasma** real time PCR Kit contains specific primers and probes labelled with a fluorescent dye for the analysis of the DNA of *Mycoplasma pneumoniae* isolated or released from clinical specimens (throat swabs, nasal swabs, bronchial lavage).

The detection of the amplification is carried out in real time via hybridization and subsequent hydrolysis of the pathogen-specific fluorescent probes. The fluorescence is measured in the FAM channel.

Furthermore, the **diarellaMycoplasma** real time PCR Kit contains a Control DNA (K4), which is detected in a second amplification system.

Added during DNA extraction, the Control DNA (K4) allows not only for the detection of PCR inhibition but also detects possible mistakes during DNA

extraction. This greatly reduces the risk of false-negative results. The amplification of the Control DNA (K4) is meassured in the VIC®/HEX/JOE™/TET channel.

## 10 Equipment and Reagents to be Supplied by User

- DNA isolation kit (e.g. **NukEx Pure** RNA/DNA, gerbion Cat. No. G05004) or **NukEx PLUS** Nucleic Acid Release Reagent, gerbion Cat. No. G01073
- Sterile microtubes
- Pipets (adjustable volume)
- Sterile pipet tips with filter
- Table centrifuge
- Vortexer
- real time PCR instrument
- Optical PCR reaction tubes with lid
- Optional: Liquid handling system for automation

## 11 Important Notes

- The **diarellaMycoplasma** real time PCR must be performed by qualified personnel only.
- Good Laboratory Practice (GLP) has to be applied.
- All samples must be regarded as potentially infectious material and all equipment used has to be treated as potentially contaminated.

## 12 General Precautions

- Stick to the protocol described in the Instruction Manual.
- Set up different laboratory areas for the preparation of samples and for the set up of the PCR in order to avoid contaminations.
- Pipettes, tubes and other materials must not circulate between those different laboratory areas.
- Always use filter tips.
- Regulary decontaminate equipment and benches with ethanol-free decontaminant.
- Do not combine **diarellaMycoplasma** real time PCR Kit components of different lot numbers.

## 13 Isolation of DNA

The **diarellaMycoplasma** real time PCR is suitable for the detection of *Mycoplasma pneumoniae* DNA isolated or released from clinical specimens (throat swabs, nasal swabs, bronchial lavage) with appropriate isolation methods.
Commercial kits for DNA isolation are recommended, e.g. :

- **NukEx Pure** RNA/DNA (gerbion Cat. No. G05004)

Alternatively DNA can be released from throat or nasal swabs with **NukEx PLUS** Nucleic Acid Release Reagent (gerbion Cat. No. G01073). This is the fastest and most convenient method for the release of nucleic acid from swabs, because column based purification of the DNA can be omitted. More information can be found on www.gerbion.com.

**Important:** In addition to the samples always run a „water control" in your extraction, possible contaminations during DNA extraction will be detectable. Treat this water control analogous to a sample.

**Please note the chapter ‚Control DNA' on page 6.**

If the real time PCR is not performed immediately, store extracted DNA according to the instructions given by the DNA extraction kit's manufacturer.
Further information about DNA isolation is to be found in the extraction kit manual or from the extraction kit manufacturer's technical service.

## 14 Control DNA (K4)

The **diarellaMycoplasma** real time PCR Kit contains a Control DNA (K4) which allows the user to control the DNA isolation procedure and to check for possible real time PCR inhibition.

Control DNA (K4) used as Extraction Control:
**diarellaMycoplasma** Control DNA (K4) is added prior to the DNA extraction.
To this end, multiply the buffer volume needed per extraction with the number of samples (including at least one water control) (N) plus 1 to compensate for inaccuracies in pipetting (N+1). Add 5 µl Control DNA (K4) per extraction (5 µl x (N+1)). Mix well. Perform the DNA isolation according to the manufacturer's instructions.

**If the extraction protocol includes an incubation step of the sample in the first buffer, the Control DNA (K4) is to be added to each sample individually after incubation.**

**The Control DNA (K4) must not be added to the sample material directly.**

Control DNA (K4) used as Internal Control of the real time PCR :
If crude NukEx PLUS lysates are being used or control of the DNA extraction is not desired, the Control DNA (K4) can be used as Internal Control of the real time PCR only. To that end, the Control DNA (K4) is to be added directly to the real time PCR Master Mix. Set up the real time PCR according to protocol B.

# 15 Real time PCR

## 15.1 Important Points Before Starting:

- Please pay attention to the ‚Important Notes' on page 5.
- Before setting up the real time PCR familiarise yourself with the real time PCR instrument and read the user manual supplied with the instrument.
- The programming of the thermal profile should take place before the PCR set up.
- In every PCR run at least one Positive Control (K2) and one Negative Control (K3) should be included.
- Before each use, all reagents should be thawed completely at room temperature, thouroughly mixed (do NOT vortex the Reaction Mix (K1) but mix by pipetting up and down repeatedly), and centrifuged very briefly. Then place all reagents on ice or on a cooling block (+2 to +8°C).

## 15.2 Procedure

If the Control DNA (K4) is used to control both the real time PCR and the DNA isolation procedure, please follow protocol A. If the Control DNA (K4) is solely used to detect possible inhibition/failure of the real time PCR, please follow protocol B

Protocol A
**The Control DNA (K4) was added during DNA extraction (see ‚Control DNA', page 6). In this case, prepare the Master Mix on ice or in a cooling block (+2 to +8°C) according to Table 2.**
The Master Mix contains all of the components needed for PCR except the sample. Prepare a volume of Master Mix for at least one sample more than required, in order to compensate for pipetting inaccuracy.

Table 2: Preparation of the Master Mix (Control DNA (K4) was added during DNA extraction)

| Reaction Volume | Master Mix Volume |
|---|---|
| 16.0 µl Reaktion Mix (K1) | 16.0 µl x (N+1) |
| 0.0 µl Control-DNA (K4) | 0.0 µl x (N+1) |

Protocol B

The Control DNA (K4) is used for the control of the real time PCR only (see ‚Control DNA', page 6). In this case, prepare the Master Mix on ice or in a cooling block (+2 to +8°C) according to Table 3.

The Master Mix contains all of the components needed for PCR except the sample. Prepare a volume of Master Mix for at least one sample more than required, in order to compensate for pipetting inaccuracy.

Table 3: Preparation of the Master Mix (Control DNA (K4) is added directly to the Master Mix)

| Reaction Volume | Master Mix Volume |
|---|---|
| 16.0 µl Reaktion Mix (K1) | 16.0 µl x (N+1) |
| 0.5 µl Control DNA (K4)* | 0.5 µl x (N+1)* |

*The increase in volume caused by adding the Control DNA (K4) is not taken into account when preparing the PCR assay. The sensitivity of the detection system is not impaired.

Protocol A and B: real time PCR set up

- Put the number of optical PCR reaction tubes needed into the cooling block.
- Pipet **16 µl** of the Master Mix into each optical PCR reaction tube.
- Add **4 µl** of the eluates from the DNA isolation (including the eluate of the water control) or the crude NukEx PLUS lysates, the Positive Controls (K2), and the Negative Control (K3) to the corresponding optical PCR reaction tube (Table 4).
- Close the optical PCR reaction tubes immediately after filling in order to reduce the risk of contamination.

**Table 4**: Preparation of the real time PCR

| Component | Volume |
|---|---|
| Master Mix | 16.0 µl |
| Sample | 4.0 µl |
| Total Volume | 20.0 µl |

For the real time PCR use the thermal profile shown in Table **5.**

**Table 5:** real time PCR thermal profile

| Discription | Time | Temperature | Number of Cycles |
|---|---|---|---|
| *Initial Denaturation* | 5 – 15 min* | 95°C | 1 |
| *Amplification of DNA* | | | |
| Denaturation | 10 sec | 95°C | 45 |
| Annealing and Extension | 40 sec<br>Aquisition at the end of this step | 60°C | |

*When using crude **NukEx PLUS** lysates that have not been heat inactivated or purified, the initial denaturation has to be increased to 15 min. For purified DNA samples 5 min denaturation is sufficient.

# 16 Data Analysis

The *Mycoplasma pneumoniae* specific amplification is measured in the FAM channel. The amplification of the Control DNA (K4) is measured in the VIC®/HEX/JOE™/TET channel.

**Following results can occur:**

- **A signal in the FAM channel is detected:**
  **The result is positive, the sample contains *Mycoplasma pneumoniae* DNA.**

  In this case, detection of a signal of the Control DNA (K4) in the VIC®/HEX/JOE™/TET channel is inessential, as high concentrations of bacterial DNA may reduce or completely inhibit amplification of the Control DNA (K4).

- **No signal in the FAM channel, but a signal in the VIC®/HEX/JOE™/TET channel is detected:**
  **The result is negative, the sample does not contain *Mycoplasma pneumoniae* DNA.**

  The signal of the Control DNA (K4) excludes the possibilities of DNA isolation failure (in case the Control DNA (K4) is being used as an Extraction Control) and/or real time PCR inhibition. If the $C_T$ value of a sample differs significantly from the $C_T$ value of the water control, a partial inhibition occured, which can lead to negative results in weak positive samples (see „Troubleshooting“, page 12).

- **Neither in the FAM nor in the VIC®/HEX/JOE™/TET channel a signal is detected:**
  **A diagnostic statement cannot be made.**

  The DNA isolation was not successful or an inhibition of the PCR has occurred. In case the Control DNA (K4) was added during DNA isolation and not directly to the PCR Master Mix, the Negative Control (K3) is negative in both channels.

Figure 1 and Figure 2 show examples for positive and negative real time PCR results.

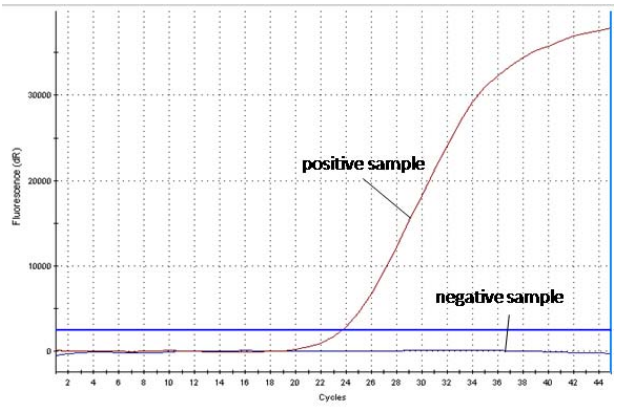


**Figure 1:** The positive sample shows bacteria specific amplification in the FAM channel whereas no fluorescence signal is detected in the negative sample.

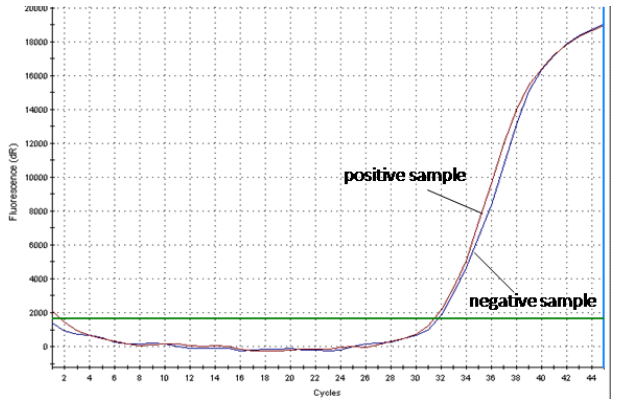


**Figure 2:** The positive sample as well as the negative sample show a signal in the Control DNA-specific VIC®/HEX/JOE™/TET channel. The amplification signal of the Control DNA (K4) in the negative sample shows, that the missing signal in the bacteria-specific FAM channel is not due to PCR inhibition or failure of DNA isolation, but that the sample is a true negative.

# 17 Troubleshooting

The following troubleshooting guide is included to help you with possible problems that may arise when performing a real time PCR. If you have further questions, please do not hesitate to contact our scientists on info@gerbion.com.

| **No fluorescence signal in the FAM channel of the Positive Control (K3)** | |
|---|---|
| The selected channel for analysis does not comply with the protocol | Select the FAM channel for analysis of the *Mycoplasma pneumoniae* specific amplification and the VIC®/HEX/JOE™/TET channel for the amplification of the Control DNA (K4). |
| Incorrect configuration of the real time PCR | Check your work steps and compare with ‚Procedure' on page 7). |
| The programming of the thermal profile is incorrect | Compare the thermal profile with the protocol (Table 5, page 9). |
| Incorrect storage conditions for one or more kit components or kit expired | Check the storage conditions and the date of expiry printed on the kit label. If necessary, use a new kit and make sure kit components are stored as described in ‚Transport and Storage', page 3 |
| **Weak or no signal of the Control DNA (K4) and simultaneous absence of a signal in the bacteria specific FAM channel** | |
| real time PCR conditions do not comply with the protocol | Check the real time PCR conditions (page 7). |
| real time PCR inhibited | Make sure that you use an appropriate isolation method (see ‚Isolation of DNA', page 6) and follow the manufacturer's instructions. Make sure that the ethanol-containing wash buffer of the isolation kit has been completely removed. An additional centrifugation step at high speed is recommended before elution of the DNA. Dilute NukEx PLUS lysates 1:3 in $_{d}H_2O$ or NukEx Universal Dilution Buffer (gerbion, Cat. No. G01014). Alternatively, purify the |

| | |
|---|---|
| | lysates with e.g. NukEx Pure RNA/DNA Kit (gerbion, Cat. No. G05004). |
| Initial denaturation too short | When using crude NukEx PLUS lysates the initial denaturation step must be performed for 15 minutes in order to heat inactivate the enzymatic component of NukEx PLUS. |
| DNA loss during isolation process | In case the Control DNA (K4) was added during extraction, the lack of an amplification signal can indicate that the DNA isolation was not successful. Make sure that you use an appropriate isolation method (commercial kits are recommended) and stick to the manufacturer's protocol. |
| Incorrect storage conditions for one or more components or kit expired | Check the storage conditions and the date of expiry printed on the kit label. If necessary, use a new kit and make sure kit components are stored as described in ‚Transport and Storage', page 3. |
| **Detection of a fluorescence signal in the FAM channel of the Negative Control (K3)** | |
| Contamination during preparation of the PCR | Repeat the real time PCR in replicates. If the result is negative in the repetition, the contamination occurred when the samples were pipetted into the optical PCR reaction tubes. Make sure to pipet the Positive Control (K4) last and close the optical PCR reaction tube immediately after adding the sample. If the same result occurs, one or more of the kit components might be contaminated. Make sure that work space and instruments are decontaminated regularly. Use a new kit and repeat the real time PCR. |

# 18 Other Products

A number of products related to real time PCR and nucleic acid isolation is available from gerbion GmbH & Co. KG. More information as well as the complete Product Catalogue is available on www.gerbion.com.

| Product | Description | Cat. No.. |
|---|---|---|
| NukEx Pure RNA/DNA | Spin column-based kit for the isolation of RNA and DNA from a variety of sample matrices. For 50 or 200 extractions. | G05004-50<br>G05004-200 |
| NukEx PLUS | Reagent for the enzymatic release of nucleic acids from swabs and cell culture suspensions.<br>Very fast and convinient protocol! | G01073 |
| NukEx Collection Tubes | 500 NukEx Collection Tubes for use with NukEx Spin Columns. | G06008 |
| NukEx Universal Dilution Buffer | Diluent for samples for real time (RT-) PCR. | G01014 |
| NukEx Pestle 1.5 ml | 100 disposable PBTP pestles for use in 1.5 ml reaction tubes. Individually packed. DNase-free, RNase-free, non-pyrogenic. | G06006 |
| NukEx TS | Shredding material aliquoted in 1.5 or 2.0 ml safe lock tubes or 2.0 ml screw cap tubes for the manual or automated preparation of samples such as tissue or insects. | G06007-1.5<br>G06005-2.0<br>G06005-2.0 sc |
| Proteinase K | Proteinase K from *Tritirachium album*. 100 mg. | G07001 |
| DNase I | DNase I, RNase-free is an endonuclease that digests single- and double-stranded DNA. It hydrolyzes phosphodiester bonds producing mono- and oligodeoxyribo-nucleotides with 5'-phosphate and 3'-OH groups.<br>10,000 U in solution (10 U/µl) supplied with 10 x DNase I Reaction Buffer. | G07002 |